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I. LOW NICOTINE STUDY

A. **Objective**: To investigate the biochemistry of the nicotine biosynthetic pathway at putrescine N-methyltransferase (PMT) and specifically to isolate PMT from tobacco root extracts.

B. **Status**: Harvesting of the group 12 tobacco plants (35 plants, with an average root weight of 450 g) was completed, and the processing to the 40-65% ammonium sulfate stage of the root extracts from group 11 was done.

A number of column sequences were examined to determine changes in specific activity and yields (2,3,4,6). With the phenyl-Sepharose, DEAE-Sepharose and Sephacryl columns, a 20 to 30-fold increase in specific activity from the ammonium sulfate stage was observed for the series; losses in PMT activity during the purification may be responsible for the low fold increases.

To reduce some of the pressure problems associated with the presence of glycerol and the routine operation of the columns, a PMT sample was applied to the phenyl-Sepharose column in the absence of glycerol. Surprisingly, PMT activity was eluted throughout the 1.5 M to 0 M NaCl gradient in the absence of glycerol rather than towards the end of the gradient as was observed when glycerol was present. An increase in specific activity of 9.9 for this step alone over the ammonium sulfate stage was seen; however, the large volume (1 L gradient) of the sample obtained by this method warrants further examination. A large, non-PMT, 280 nm absorption peak normally observed with the phenyl-Sepharose column in the presence of glycerol was still present in this glycerol-free column. This indicated that the absence of glycerol allowed the separation of PMT from this non-PMT, 280 nm absorbing specie(s). Maximization of this anomalous behavior to enhance the purification is being addressed.

Because of the above observations, a purification scheme was attempted with the complete absence of glycerol at all stages. PMT-activity decreased dramatically unless tha samples were concentrated against sucrose during the interim steps, i.e., stabilized by an osmoticum.

Studies addressing the stability of PMT have been implemented because of the rapid decline in PMT-activity in the absence of glycerol. Glycerol was added back to a PMT sample which had been subjected to Sephadex G-25 chromatography to remove glycerol and salts. The following glycerol concentrations were examined 20, 10, 5, 1 and 0% (v/v) over a total of five days. Twenty per cent glycerol stabilized the PMT activity of these samples up to 48 hr

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at 4°C. At 48 hr, the PMT activities for the other samples (1, 5, and 10% glycerol) were less than 50% of the 0 time samples. The PMT activity for the 20% glycerol sample was reduced to about 50% of the 0 time sample in five days, whereas the PMT activities were less than 20% of the 0 time samples by the fifth day for the other glycerol concentrations tested. Different salt concentrations were also examined to determine the efficacy of salt stabilization (0, 0.05, 0.10, 0.25 and 0.50 M KCl). After 24 hr, all samples showed PMT activities approximately ≤ to 50% of the 0 time samples. Both of these studies showed that of the conditions examined, PMT-containing fractions were stable only in 20% glycerol.

To characterize further the enzyme that is being isolated as PMT, substrate specificity studies were implemented. The substrates chosen were the structurally similar diamino-analogs of putrescine (diaminobutane), diaminopropane and diaminopentane. The PMT activities of the analogs compared to the controls with putrescine averaged 2.6% for diaminopropane and 1.1% for diaminopentane. The starting material for these studies came from two different PMT-containing preparations obtained through DEAE-Sepharose and phenyl-Sepharose columns. These results lend support to the notion that the enzyme that is being isolated is indeed PMT.

PMT was also subjected to SDS-PAGE in the presence and absence of 2-mercaptoethanol to examine the monomeric nature of PMT. Under these conditions, only minor changes in the polypeptide banding pattern were observed in regions other than the 55-65 kDalton region indicating the monomeric nature of PMT (3).

A preparative isoelectric focusing (IEF) device was also examined (Rotofor, BioRad) for possible use in the purification of PMT. Peak PMT activity was obtained in a fraction focusing around pH 5.1. The SDS-PAGE profile indicated possible aggregation which may require the use of detergents for dissolution (3). To examine the possibility that Triton X-100® could be used for this purpose, PMT active samples were incubated with the IEF solution and Triton X-100®. The PMT activities were found to decrease approximately 30% to 40% (0.1 and 1% Triton X-100®, respectively) from the control over a time period similar to that used during the isoelectric focusing. This suggests that Triton® may be useful during the isoelectric focusing step.

Immunochemical studies were continued with the MagniSort beads (DuPont) (5). Some decrease in PMT-activity was observed for four antibody samples obtained from Hazleton even after considering that seen with the controls (5).

C. **Plans**: Extracts from the group 12 harvest will be processed to the 40-65% ammonium sulfate stage. Modification of the conditions to elute PMT from phenyl-Sepharose in the absence of glycerol will be continued. Examination of larger sample sizes and alternate pooling practices for the Sephacryl column will be examined with the aim to increase the specific activity towards 1000 from the

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ammonium sulfate stage. Further stability studies using polyethylene glycol and ethylene glycol will be examined. These may assist in the elution of PMT from the hydrophobic interaction column (phenyl-Sepharose) and stabilize PMT for further column examination (e.g., Sepharose gel filtration column). Other substrates for PMT will be tested and Km data will be obtained for putrescine and SAM.

D. References:

1. Dunn, R. L. Notebook No. 7899.
2. Malik, V. Notebook No. 8542.
3. Sykes, A. Notebook No. 8526.
4. Yu, T. Notebook No. 8381.
5. Mooz, E. D. Notebook No. 8599.
6. Crockett, E. Notebook No. 8563.

II. ALTERNATE HUMECTANTS (PG/G-FREE SHEETS/CIGARETTES) (1)

A. Objective: To produce an acceptable cigarette (domestic Marlboro-type) which is PG/G-free by the end of the third quarter of 1988.

B. Results: 100% test cigarettes made from production plant PG/G-free RLTC and RL150B sheets were the preferred choice when compared to 100% control RL cigarettes (2). Subjective data from 100% test cigarettes made from production plant PG/G-free RCB and control RCB are being reevaluated (2). Samples of PG/G-free, RL cigarettes submitted for toxicological evalution were found to be similar to the controls (3). PG/G-free RCB and control cigarettes were retested and found to give similar toxicological results (3).

C. Plans: Zero time survivability tests on the production plant PG/G-free, RL and RCB sheets will be made. Small-scale, blended PG/G-free cigarettes and control cigarettes will be made in the Semi-works for subjective and toxicological evaluations.

D. References:

1. Mooz, E. D. Notebook No. 8599.
2. Ruziak, S. Personal communication. 1988 June 17.
3. Thompson, L. Personal communication. 1988 June 24.

III. ALTERNATE HUMECTANTS (GLYCERINE-FREE SHEET/CIGARETTES) (1)

A. Objective: To produce an acceptable glycerine-free (G-free) cigarette (domestic Marlboro-type) for a POL test by the end of the third quarter of 1988.

B. Results: Data are being obtained from the zero time survivability test run in the Semi-works on G-free RCB sheets made at the BL Plant in 2/88. Information from the three-month, G-free, Park 500 RL sheet survivability test have been received and are being evaluated.

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Control and G-free POL cigarettes made in April were rejected due to three factors: 1) a possible problem with the tar delivery data on the control cigarette (2); 2) low total PG and G levels in the control (2); and 3) the unacceptable subjectives from the Richmond Panel regarding both the test and control cigarettes (3).

C. **Plans**: A G-free, POL remake of Marlboro and control cigarettes is scheduled in the Semi-works for the week of July 11.

D. **References:**

1. Mooz, E. D. Notebook No. 8599.
2. Ruziak, S. Personal communication. 1988 April 22.
3. Spielberg, H. Personal communication. 1988 April 29.

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